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**14. ABSTRACT**

We investigated a novel chondroitin sulfate-based mechanism of tumor-stromal communication and regulation of the canonical Wnt pathway in breast cancer progression. We hypothesized that elevated expression of the Chondroitin-4-sulfotransferase-1 gene (C4ST-1) in tumor-associated stroma represents a novel control mechanism that could enhance canonical Wnt signaling in breast cancer cells through the establishment of a microenvironment enriched in CS-E, a chondroitin sulfate product of C4ST-1. Our research showed that the chondroitin sulfate form CS-E could inhibit canonical Wnt signaling. Moreover, CS-E was able to interfere with biological outcomes of Wnt signaling, including proliferation and invasiveness of breast cancer cells. Our data suggest that stromally-derived CS-E could interfere with Wnt-driven oncogenic effects in breast cancer cells. Thus, these results lay the groundwork for future studies to investigate the pharmacological exploitation of CS-E as an intervention of Wnt-driven breast cancer.

**15. SUBJECT TERMS**
chondroitin sulfate; C4ST-1; tumor stroma; Wnt/beta-catenin signaling

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INTRODUCTION

Molecules of the tumor microenvironment play a critical role in tumor progression [1-4]. The proteoglycan chondroitin sulfate, and chondroitin sulfotransferase genes controlling its sulfation fine structure, has been suggested to play critical roles in many aspects of health and disease [5-10]; however, its role in breast cancer progression are not well understood. We investigated a novel chondroitin sulfate-based mechanism of tumor-stromal communication and regulation of the canonical Wnt pathway in breast cancer progression. We hypothesized that elevated expression of the *Chondroitin-4-sulfotransferase-1* gene (*C4ST-1*) in tumor-associated stroma represents a novel control mechanism that could enhance canonical Wnt signaling in breast cancer cells through the establishment of a microenvironment enriched in CS-E, a chondroitin sulfate product of C4ST-1. For this, we wanted to firstly determine the functional significance of stromal C4ST-1 and CS-E on the behavior of, and canonical Wnt signaling in, co-cultured breast cancer cells *in vitro*. Secondly, we wanted to determine the functional role of stromal C4ST-1 and CS-E on breast cancer progression and canonical Wnt signaling *in vivo.*

BODY

1) **Specific Aim 1**. We wanted to determine the functional significance of stromal C4ST-1 and CS-E on the behavior of, and canonical Wnt signaling in, co-cultured breast cancer cells *in vitro*.

   *Task 1a). Generation of Balb/c-3T3 fibroblasts stably carrying C4ST-1 overexpression or shRNA as well as GFP cassettes; generation of mCherry-tagged EMT6 cells (available in our lab) expressing a β-catenin S33Y cDNA, generation of luciferase-tagged EMT6 cells (available in our lab) expressing a β-catenin S33Y cDNA. This will create the cell lines necessary for our proposal.*

   Accomplished: We encountered problems with viral transductions in the generation of Balb/c-3T3 fibroblasts stably overexpressing a C4ST-1 cDNA. Specifically, transduced and selected pools of cells did not show overexpression of C4ST-1 (data not shown). We therefore used stable transfection of a pCAGIP-C4ST-1 plasmid (or pCAGIP plasmid without insert as control to generate cells overexpressing C4ST-1 (Figure 1). We utilized Balb/c-3T3-pCAGIP clones #2 and #5, and Balb/c-3T3-pCAGIP-C4ST-1 clones #5 and #6 for the studies below. We generated Balb/c-3T3 cells stably expressing mCherry (Balb/c-mCherry) and EMT6 cells stably expressing GFP (EMT6-GFP) and a β-catenin S33Y cDNA (EMT6-b-catS33Y). We generated EMT6 cells stably expressing Firefly luciferase (EMT6-FFluc; data not shown). However, in the studies below we sometimes encountered problems getting Wnt3a stimulation in TOPFLASH assays with these EMT6 cells. Therefore, we also generated mouse 4T1 breast cancer cell clones stably expressing FFluc (4T1-FFluc; dat anot shown). 4T1 breast cancer cells, like EMT6 breast cancer cells, are derived from Balb/c mice, and have also been used extensively in orthotopic mouse breast cancer studies. These cells showed a consistent and strong response to Wnt3a stimulation in TOPFLASH assays, and where used subsequently for *in vivo* and some *in vitro* studies. We also generated two different stable C4ST-1-shRNA clones; however, C4ST-1 expression was not affected by either shRNA (data not shown); therefore, we could not assess the effects of loss of C4ST-1 expression in the tasks below.

*Task 1b). 2D co-culture of Balb/c-3T3 stromal fibroblasts generated in 1a) and EMT6 breast cancer cells, treatments with chondroitin sulfates and Chondroitinase ABC, analysis of EMT6 cell proliferation, apoptosis, canonical Wnt signaling. This will establish whether stromal C4ST-1 and chondroitin sulfates participate in stromal-tumor communication in vitro.*

Accomplished: We encountered problems in the co-culture of Balb/c-3T3 cells with EMT6 or 4T1 breast cancer cells. Specifically, EMT6 and 4T1 cells could not adhere to cultured Balb/c-3T3 fibroblasts *in vitro*. Previous reports have suggested that normal fibroblasts need to be "transformed" by treatment with conditioned media from breast cancer cells in order to support cancer cell attachment and growth. Therefore, we treated Balb/c-3T3 fibroblasts for 6 days with conditioned media from EMT6 cells. However, even after this treatment, EMT6 and 4T1 breast cancer cells could not adhere to these fibroblasts. This problem persisted in the presence or absence of C4ST-1 overexpression in Balb/c-3T3 cells. Due to these difficulties, we were not able to complete the co-culture aspects of this aim.

We next determined the effects of treatment with differently sulfated chondroitin sulfate forms (C4S, C6S, CS-D, or CS-E) on proliferation, apoptosis, and canonical Wnt signaling in EMT6 and 4T1 breast cancer cells. We analyzed proliferation by EdU incorporation and detection methods. Our data showed that treatment with CS-E at 100μg/ml could significantly interfere with Wnt3a-stimultated proliferation (Fig.1). This inhibitory effect was not observed with treatment with the chondroitin sulfate forms C4S, C6S, or CS-D, or enzymatic elimination of breast cancer cell-associated chondroitin sulfates by treatment with the bacterial enzyme Chondroitinase ABC (ChABC) (Fig.1). We analyzed apoptosis by TUNEL staining. No cells undergoing apoptosis were detected in any of the treatments (data not shown). We analyzed canonical Wnt signaling by TOPFLASH reporter assays and immunofluorescence detection of β-catenin. These experiments showed that treatment with exogenous CS-E at 100μg/ml, but not with C4S, C6S, CS-D or ChABC, could inhibit canonical Wnt signaling in breast cancer cells (Fig.2). The effect of CS-E on canonical Wnt signaling was dose-dependent, with little inhibition seen at 5μg/ml, and maximum effect observed at 100μg/ml (data not shown).

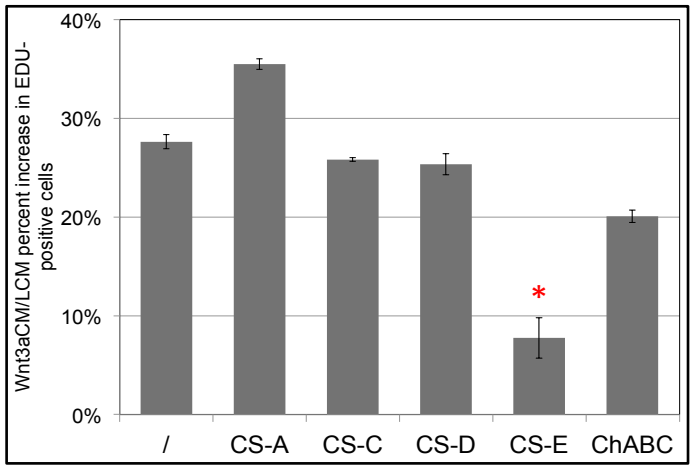
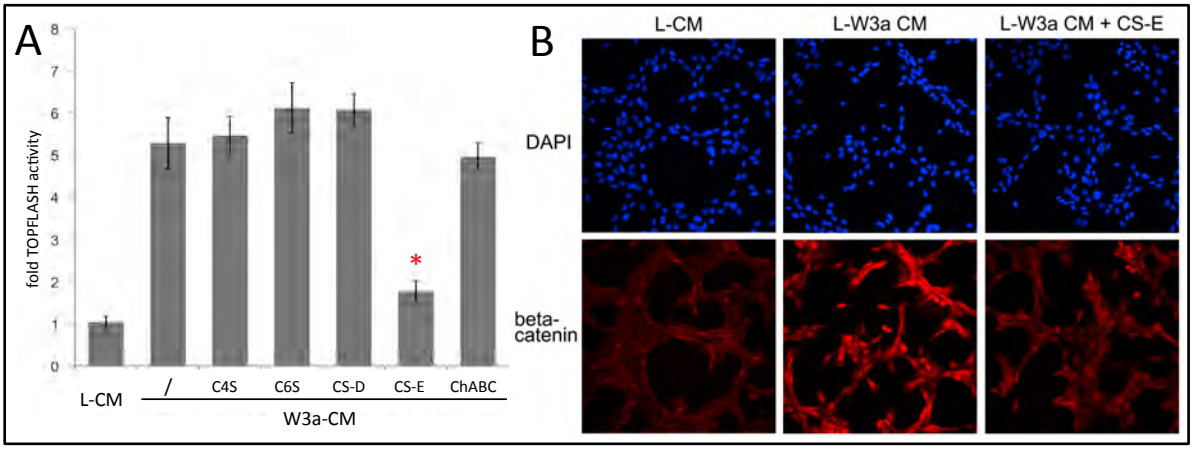



**Figure 1. Effects of chondroitin sulfates and ChABC treatment on Wnt3a-stimulated proliferation.** EMT6 cells were treated with Wnt3a in the presence or absence of C4S, C6S, CS-D, CS-E or ChABC. Proliferation was detected by EdU incorporation and detection, followed by quantitation of EdU-positive nuclei/DAPI-stained nuclei. CS-E was able to reduce Wnt3a-induced proliferation significantly (*; P<0.05).

**Figure 2 (next page). CS-E, but not other chondroitin sulfate forms, or elimination of cell-associated chondroitin sulfate by treatment with ChABC, can interfere with Wnt3a-induced TOPFLASH reporter activity (A) and β-catenin accumulation and nuclear translocation (B) in EMT6 cells.** Scale bar in d = 30μm.




*Task 1c). 3D Matrigel co-culture of Balb/c-3T3 stromal fibroblasts generated in 1a) and EMT6 breast cancer cells, analysis of proliferation, apoptosis, invasiveness, and canonical Wnt signaling. This will establish whether stromal C4ST-1 and chondroitin sulfates participate in stromal-tumor communication in an in vivo-like Matrigel environment.*

Accomplished: We again encountered problems in the co-culture of Balb/c-3T3 cells with EMT6 or 4T1 breast cancer cells, as described above. Due to these difficulties, we were not able to complete the co-culture aspects of this aim. We next looked at the influence of exogenous chondroitin sulfates on proliferation, apoptosis, invasiveness, and canonical Wnt signaling in EMT6 cells in 3D cultures. Treatment with CS-E, but not other chondroitin at 100µg/ml had no effect on proliferation, as determined by EdU incorporation and staining, followed by quantitation of EdU-positive nuclei to DAPI-stained nuclei (Fig.3). We next analyzed apoptosis by TUNEL staining; we did not observe any TUNEL positive cells with or without CS-E treatment in 3D cultures (data not shown). To study invasiveness, we analyzed and quantified the formation of invasive protrusions from EMT6 3D multicellular structures into the surrounding matrigel. Treatment with exogenous CS-E at 100µg/ml, but not C4S, C6S, CS-D, or ChABC, caused a striking

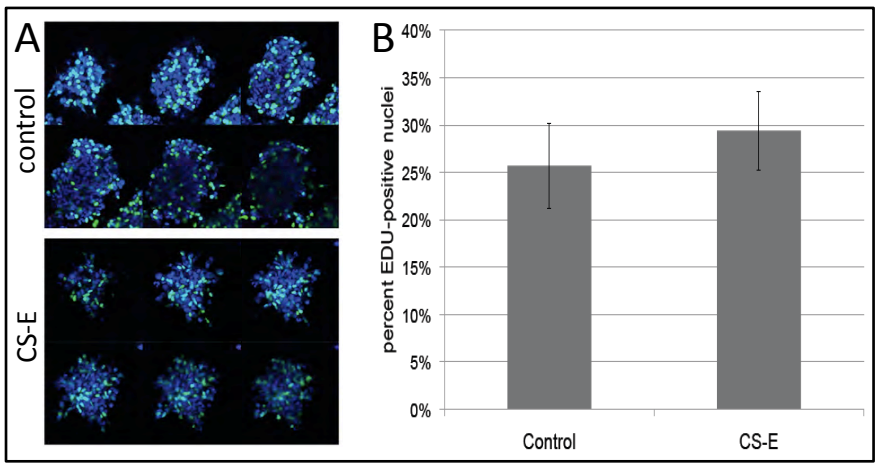



**Figure 3. Effect of CS-E on proliferation in EMT6 3D cultures.** (A) EdU staining of confocal Z-stacks of control and CS-E treated 3D cultures. EdU=green; DAPI=blue. (B) Quantitation of EdU-positive cells showed no significant differences between controls and CS-E treatment.

decrease in invasive protrusion formation (Fig.4A). This effect of CS-E showed a clear dose-dependency (Fig.4B-F).

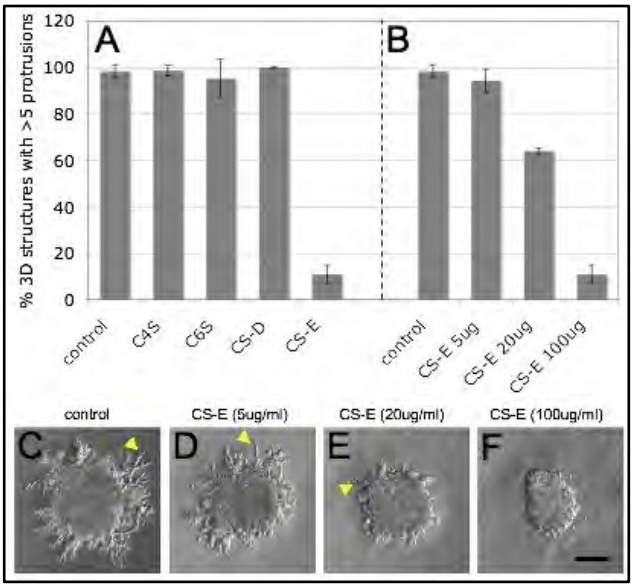



**Figure 4. Exogenous CS-E impairs invasiveness of EMT6 cells in 3D cultures.** (A) Treatment with exogenous CS-E, but not other chondroitin sulfates forms, led to a significant reduction in invasive protrusion formation in EMT6 3D cultures (statistical significance: P<0.001). (B-F) Treatment of EMT6 cells with increasing doses of exogenous CS-E demonstrated a dose-dependent effect, with little loss of protrusions observed at 5ug/ml CS-E (B,D), and an almost complete absence of protrusions at 100μg/ml (B,F). Scale bar in (F) = 100μm.

In order to analyze the effect of CS-E treatment on canonical Wnt signaling, we analyzed expression of β-catenin in lysates of control and CS-E (100μg/ml) - treated EMT6 3D cultures by Western blot (Fig.5). This analysis showed that CS-E treatment leads to a reduction in β-catenin levels. At the same time, CS-E treatment did not affect other signaling pathways, including TGFβ (phospho-Smad2/Smad2/3) or MAP Kinase signaling pathways (phospho-Erk/Erk; phospho-p38/p38) (Fig.5). These results demonstrate that CS-E treatment can specifically interfere with canonical Wnt signaling in EMT6 3D cultures. Moreover, this task revealed that CS-E could interfere with specific cellular responses typically associated with active canonical Wnt signaling, including increased invasiveness, while not affecting other cellular responses to Wnt (proliferation).

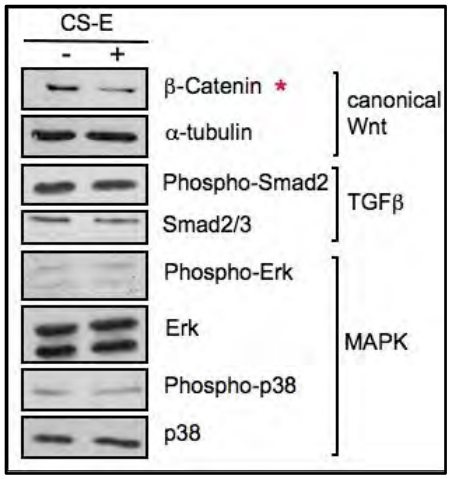



**Figure 5. Treatment of EMT6 3D cultures with CS-E leads to a reduction in β-catenin levels, while not affecting TGFβ or MAPK signaling pathways.**

*Task 1d). 2D and 3D Matrigel culture of Balb/c-3T3 stromal fibroblasts and EMT6- β-catenin S33Y breast cancer cells generated in 1a), treatments with chondroitin sulfates and Chondroitinase ABC, analysis of EMT6 cell proliferation, apoptosis, canonical Wnt signaling, and invasiveness. This will establish whether stromal C4ST-1 and chondroitin sulfates affect co-cultured EMT6 cells solely through the canonical Wnt pathway in vitro.*

Accomplished: We encountered problems in the co-culture of fibroblasts with EMT6 and 4T1 breast cancer cells. Thus, this task was not completed.

2) **Specific Aim 2**. We wanted to establish the functional role of stromal C4ST-1 and CS-E on breast cancer progression and canonical Wnt signaling *in vivo.*

The *in vivo* aims of this application where delayed due to necessary repairs of our Xenogen IVIS-200 *in vivo* luminescence imaging system. We were granted a no-cost extension to be able to get the instrument repaired in time. This extension allowed us to complete most of the animal procedures of our application; however, post-experimental data analysis and histological analysis of tumors and tissues with metastasis has not been performed yet.

*Task 2a). Orthotopic injection of Balb/c-3T3 stromal fibroblasts generated in 1a) and luciferase-tagged EMT6 breast cancer cells (available in the lab) into Balb/c female mice, treatment with chondroitin sulfates, analysis of primary tumor growth and metastases. We will use 144 mice for this sub-aim. This will establish whether stromal C4ST-1 and chondroitin sulfates affect breast cancer progression in vivo.*

Accomplished: In order to evaluate the effects of C4ST-1 expression in stromal compartments on tumor progression, we generated orthotopic breast cancer mouse models utilizing co-implantation of 4T1-FFluc cells with Balb/c-3T3-pCAGIP-C4ST-1 cells, or with Balb/c-3T3-pCAGIP cells as controls. We utilized two clones for each condition. In a different experiment, we evaluated the effects of CS-E treatment on breast tumor progression. For this, we generated orthotopic breast tumors using our 4T1-FFluc cells. When tumors became palpable, we performed bi-daily IP injections of CS-E. As controls, we injected either water or C4S, which did not affect canonical Wnt signaling or breast cancer cells in 3D cultures [see task 1b) and 1c)]. Tumor progression and metastasis was followed *in vivo* by luminescence detection of 4T1-FFluc cells using a Xenogen IVIS-200 system. These orthotopic tumor experiments were completed, but due to the delay caused by the repair of the Xenogen imaging system, the data analysis has not yet been completed. Very preliminary data analysis might suggest an inhibitory effect of CS-E treatment on tumor progression and metastasis (Fig.6). Data from both experimental series will be analyzed over the next months to determine any significant effect of stromal C4ST-1 expression, or treatment with exogenous CS-E, on tumor progression and metastasis.

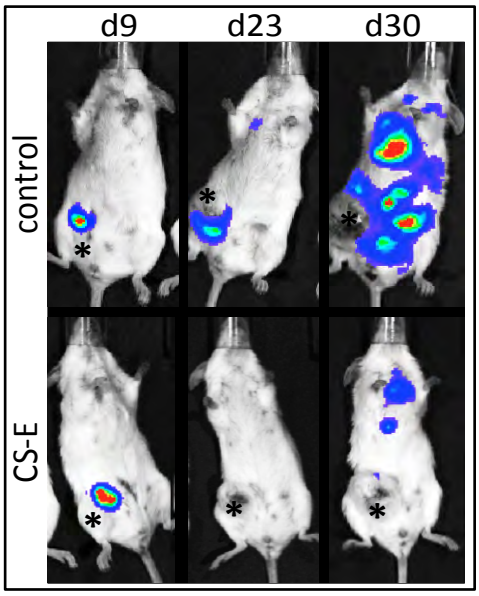



**Figure 6. CS-E treatment and 4T1 orthotopic breast cancer progression.** Examples of control mouse (upper row) and mouse injected with CS-E (lower row). Controls showed tumor progression with significant metastasis at later stages. In a subset of animals treated with CS-E, we observed loss of luminescence in the primary tumor and reduced metastasis. * = primary tumor. Days 9, 23 and 30 after orthotopic implantation are shown. Quantitative signal reference not shown.

*Task 2b). Orthotopic injection of Balb/c-3T3 stromal fibroblasts generated in 1a) and luciferase-tagged EMT6 breast cancer cells expressing a β-catenin S33Y cDNA into Balb/c female mice, treatment with chondroitin sulfates, analysis of primary tumor growth and metastases. We will use 48 mice for these experiments. This will establish whether stromal C4ST-1 and chondroitin sulfates affect breast cancer progression in vivo solely through regulation of the canonical Wnt pathway.*

Accomplished: This sub-aim could not be initiated due to the delays in the repair of our Xenogen *in vivo* imaging system.

*Task 2c). Harvested primary tumors and tissues with metastases will be sectioned and analyzed for markers of proliferation, apoptosis, and canonical Wnt signaling. This will investigate the mechanistic effects of stromal C4ST-1 and chondroitin sulfates on breast cancer progression and canonical Wnt signaling in vivo.*

Tumors and tissues with metastasis [see task 2a)] were harvested and embedded for sectioning. However, due to the delays in repair of the Xenogen system, this sub-aim has not been initiated yet. We will perform a histological analysis of tumors and tissues with metastasis in the future with financial support from our start-up account here at CMRC.

## KEY RESEARCH ACCOMPLISHMENTS

- CS-E, but not other chondroitin sulfate forms, can inhibit canonical Wnt signaling, and Wnt3a-stimulated proliferation, in EMT6 breast cancer cells *in vitro.*

- CS-E, but not other chondroitin sulfate forms, can inhibit canonical Wnt signaling, and breast cancer cell invasiveness, in EMT6 3D Matrigel cultures *in vitro.*

- Systemic delivery of CS-E *in vivo* might interfere with progression of orthotopic breast tumors.

## REPORTABLE OUTCOMES

Presentation:
Era of Hope Conference, August 2-5, 2011 [11]

Funding applied for based on work supported by this award:
NCI R01: Dissociation of oncogenic and physiological Wnt/β-catenin signaling.
Applied Feb 2012

Manuscripts:
We expect to write a manuscript(s) detailing the effects of CS-E on canonical Wnt signaling in breast cancer cells, and in orthotopic tumor models, in the near future.

## CONCLUSION

Our research showed that the chondroitin sulfate form CS-E could inhibit canonical Wnt signaling. Moreover, CS-E was able to interfere with biological outcomes of Wnt signaling, including proliferation and invasiveness of breast cancer cells. Our data suggest that stromally-derived CS-E could interfere with Wnt-driven oncogenic effects in breast cancer cells. Thus, these results lay the groundwork for future studies to investigate the pharmacological exploitation of CS-E as an intervention of Wnt-driven breast cancer.

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## APPENDICES

none

## SUPPORTING DATA

none